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C-Glycosphingolipids with an *exo*-Methylene Substituent: Stereocontrolled Synthesis and Immunostimulation of Mouse and Human Natural Killer T Lymphocytes

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Keywords

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Glycosphingolipids (GSLs) are a diverse group of lipids that control a myriad of biological and physiological processes, including stimulation of the immune system.^[1] The invariant Natural Killer T (iNKT) cells are an evolutionary conserved sub-lineage of T cells that are characterized by the expression of an invariant T cell receptor (TCR) α -chain (V α 24-J α 18 in human and V α 14-J α 18 in mouse) and reactivity to self- and microbial-derived glycolipids presented by monomorphic HLA class-I-like molecule. CD1d iNKT cells specifically recognize α -galactosylceramide (**1**, commonly referred to as α -galactosylceramide C26:0 or α -GalCer; see Scheme 1) and its analogues.^[2] When NKT cells are activated by GSL antigens presented by CD1d, they rapidly produce large amounts of immunoregulatory cytokines. The ternary complex formed by **1**, CD1d, and the iNKT cell TCR has a long half-life,^[3] which leads to unselective production of T-helper 1 (Th1) and T-helper 2 (Th2) cytokines; furthermore, overstimulation of iNKT cells by **1** results in a long-term unresponsiveness of iNKT cells.^[4] These effects have impeded therapeutic applications of **1** and have led to efforts to prepare various structural analogues that (a) selectively stimulate iNKT cells to secrete either Th1 or Th2 cytokines and (b) have a reduced affinity for the TCR to minimize iNKT cell overactivation.^[4] α -GalCer derivatives bearing a truncated phytosphingosine chain (denoted as OCH, **2a**)^[5] or a C20:2 amide chain (**2b**)^[6] have been identified as ligands that polarize iNKT cells to produce Th2 cytokines. On the other hand, Th1-polarizing ligands have been found by installation of an aromatic group at the terminus of the amide chain (such as **3**)^[7] or derivatization of the galactosyl 6-hydroxyl group.^[8]

Nevertheless, the potential clinical effectiveness of ligands such as **1**, **2a**, **2b**, and **3** may be compromised in vivo during intracellular trafficking because *O*-glycosides are prone to metabolic instability resulting from hydrolysis by either endogenous glycosidase activity or by the low pH milieu of the endosomal compartment.^[9] Therefore, C-glycoside analogues

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of **1** that are resistant to glycosidases have been developed.^[10,11] α -C-GalCer (**4**), which is resistant to in vivo degradation, was found to be more potent than **1** in mouse models of cancer and malaria.^[10] Although the TCR affinity for the CD1d-**4** complex was lower than that of the CD1d-**1** complex, the in vivo stability of **4** is postulated to be higher than that of **1**. A prolonged production of IFN γ by downstream activation of natural killer (NK) cells has been proposed to account for this bioactivity.^[12] The Th2 selectivity induced by OCH (**2a**) might also be mediated by downstream events,^[13] but the C-glycoside analogue of OCH, which was designed to be a Th2-biasing ligand with in vivo stability, failed to induce cytokines in vitro and did not elevate cytokine levels in vivo when administered intravenously to C57BL/6 mice.^[14]

Unfortunately, no significant stimulation of human iNKT cells was observed with the O-glycoside OCH (**2a**)^[6b] or C-glycoside **4**.^[15] These findings have raised the question of whether analogues identified in the mouse model as polarizing ligands of iNKT cell function will have the same or similar activities in humans.^[16] Interestingly, two analogues of **4** with structural modifications in the linker region between the hydrophilic galactosyl and the hydrophobic phytoceramide moieties were found to stimulate human iNKT cells to some extent. For example, the C-analogues **5a** and **5b**, which have an *E* double bond as a spacer, induced human iNKT cells to secrete a high level of Th1 cytokines.^[15] Also, the 1-methylene-linked C-glycoside analogue, α -1 C-GalCer (**6a**),^[17] albeit being a less potent agonist for human iNKT cells than **1**, induced higher ratios of Th1/Th2 cytokines than **1** and **4** in human iNKT cells in vitro, as evidenced by a higher ratio of both IFN γ :IL-4 and IFN γ :IL-13.^[17a] Thus, relatively subtle changes in the linker may have marked consequences with respect to the immune response, but surprisingly little attention has been devoted to the linker, especially in C-glycoside analogues, except for those mentioned above.

In this communication, we report the chemical synthesis and biological properties of **7a,b**, two novel *N*-acyl variants of C-glycosides analogues containing a 2-*exo*-methylene group in the linker area. Our synthetic route showcases the application of Nozaki-Hiyama-Kishi (NHK) chemistry^[18] for C-glycoside synthesis. The results show that the new analogues are agonists of both human and mouse iNKT cells. We found a mixed Th1/Th2 cytokine response in human iNKT cells in vitro, and a Th2-polarized cytokine response and iNKT cell expansion in mice at a concentration as low as 500 ng/mouse.

As shown in Scheme 2, our initial synthetic plan was to employ hydrozirconation-transmetalation-addition^[19] as the key step for coupling the galactosyl and lipid moieties. It is noteworthy that this method takes place under mild conditions, which might avoid the possible anomerization of **11** to the corresponding β anomer under basic conditions.^[20] The synthesis of terminal alkyne **10** commenced with the alkynylation of pentadecanal (**8**) with methyl propiolate catalyzed by the (*S,S*)-ProPhenol ligand.^[21] Subsequent hydrolysis and decarboxylation **20** provided propargylic alcohol **9** in 22% yield over three steps with 3:1 er.^[22-24] Silylation of **9** afforded TBS-protected propargylic alcohol **10** in 96% yield. Unfortunately, after **10** was hydrozirconated using the Schwartz's reagent^[25] and transmetalated with Me₂Zn,^[19] treatment of the resulting alkenylzinc of aldehyde **11** did not afford the desired allylic alcohol **12**, but instead gave complete recovery of **11**. We then turned our attention to a NiCl₂/CrCl₂-mediated coupling of aldehyde **11**^[17a] and iodoolefin **15** utilizing a reaction developed by Kishi and Nozaki et al.^[18] The synthesis of iodo olefin **15** started with 1-hexadecene (**13**), which was converted to vinyl iodide **14** in 35% yield over five steps.^[26] Then, **15** was obtained from **14** by a straightforward manipulation of protecting groups. The optimal procedure developed for the coupling of **11** with **15** entailed treatment of **11** and **15** (molar ratio 2.6:1) with NiCl₂/CrCl₂ (0.3:99.7 w/w) in THF/DMF (2:1) at rt, affording coupling product **12** in 90% yield, as roughly a 1:1 mixture of the

allylic alcohol isomers.^[27] Under these conditions, we did not detect production of the β anomer of **12**.

Dess-Martin oxidation of allylic alcohol **12** provided enone **16** in 78% yield uneventfully (Scheme 3). We next investigated methylenation of ketone **16**. We found that α to β anomerization of enone **16** occurred on Wittig methylenation, presumably via a base-induced β -elimination followed by an intramolecular hetero-Michael reaction.^[20] In a previous methylenation investigation of base-sensitive ketones, we found that Tebbe methylenation was successful.^[28] Thus enone **16** was added to 2.3 equiv of Tebbe reagent slowly at rt for 40 min, with stirring for an additional 30 min, to afford alkene **17** in 59% yield. Removal of the TBS group provided dienol **18**, which was subjected to Sharpless asymmetric epoxidation (SAE)^[29] using D-(–)-DIPT to furnish vinyl epoxide **19** (dr > 20:1). Reaction of **19** with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) gave an epoxytrichloroacetimidate, which on cyclization in the presence of Et₂AlCl^[26,30] gave dihydrooxazine **20** in 48% yield over three steps, with the required configurations in the phytoceramide moiety. Hydrolysis of **20** followed by *N*-acylation of the resulting amine with cerotyl chloride afforded amides **21a** in 42% yield for three steps. After Birch reduction of **21a**, the target compound **7a** was obtained in 73% yield.

Recent reports that *O*-glycosides with a terminal phenyl or biphenyl group in the amide chain have an enhanced IFN γ production^[7] prompted us to synthesize amide **21b** (54%, three steps) by *N*-acylation with 11-(*p*-fluorophenyl)undecanoyl chloride.^[31] The fluorine atom in the aryl group did not survive the Birch reduction, and compound **7b** was isolated in 59% yield. As the corresponding defluorinated analogue of **3** was found to be still a Th1-biased ligand,^[7] we did not pursue further efforts to install the 11-(*p*-fluorophenyl)undecanoyl chain into the new *C*-glycoside.

To examine the potential iNKT agonistic activity of the synthesized GSLs, human in vitro expanded iNKT cells were co-cultured with primary human monocytes that were pre-pulsed overnight with a high concentration (10 μ g/mL) of each GSL or with 100 ng/mL of **1** as a positive control. The iNKT cells co-cultured with vehicle pulsed monocytes served as a negative control. As depicted in Figure 1, of the seven GSLs we tested, only **7a** and **7b** induced iNKT cells to produce significant levels of IFN γ and IL-4 ($P < 0.001$). The titration of these two analogues demonstrated that IFN γ and IL-4 could be detected when the GSLs were added at 10 μ g/mL, 2 μ g/mL, or 500 ng/mL.^[32] Figure 1 also shows that *C*-glycosides **7a** and **7b** induced similar levels of IL-4 and IFN γ as found with the potent iNKT agonists **1** and **3**.^[7] In contrast, **4** and **22** failed to induce detectable levels of cytokines above the background levels in human iNKT cells, although they were found to be agonists of murine iNKT cells.^[12] It is noteworthy that ligands **6b** and **23a,b** (see Scheme 1),^[17b,31] which possess several positive modifications previously reported to trigger Th-1 selectivity in mouse splenocytes in vitro, failed to initiate a noticeable amount of cytokine production.

Because the effectiveness of the NKT cell mediated immune response mainly depends on an indirect activation of other cell types rather than the cytokine polarity of the iNKT cell itself, we next explored the in vivo activity of the *exo*-methylene-containing GSLs. Mice were injected intravenously (i.v.) with **7a,b** or with **1** as a positive control. The ligands were used at the same concentration (500 ng/mouse), which was previously found to be sufficient for maximal biological activity without toxicity for **1** in mice.^[33] Serum was collected 6 h after injection and the concentrations of IL-4 and IFN γ were measured. GSL **7a** but not **7b** induced detectable levels of cytokines (data not shown). We then conducted kinetic studies with **7a** (Figure 2). Serum was collected at 2, 6, and 24 h post i.v. injection. After the control mice were injected with 500 ng of **1**, we found that IL-4 production was the highest at the

earliest time point followed by a rapid decline by 6 h post injection; this finding is similar to published results.^[13,34] IFN γ production in the control mice increased with time, reaching the highest level at 24 h (Figure 2A). In contrast, injection of **7a** induced IL-4 formation at a slower rate, peaking at 6 h, while IFN γ production remained low at all time points tested (Figure 2B). Although **7a** induced much lower levels of cytokines than **1** at all time points, it is important to note that we discovered that **7a** induced a pronounced Th2-like response in mice. The Th2-skewed cytokine response is likely due to the inability of **7a** to induce NK cell transactivation in mice since NK cells have been shown to be the main source of IFN γ in vivo after iNKT cell activation with Th1-polarizing GSLs.^[34]

Finally, we examined whether treatment with the new iNKT cell agonists could stimulate iNKT cell proliferation in vivo. For these experiments, mice were injected with **7a**, or with **1** as a positive control or with phosphate-buffered saline (PBS) as a negative control. Three days later, splenocytes were harvested and stained for iNKT cells using the CD1d/PBS-57 tetramers.^[35] Fluorescence-activated cell sorting (FACS) plots showed that the iNKT cell population in the **7a**-treated mice was doubled compared with the negative control, although the expansion was not as large as in the **1**-treated mice (Figure S1). This indicates that the new ligand **7a** is capable of inducing iNKT cell expansion in vivo.

In summary, two new *C*-glycoside analogues of KRN7000 containing a 2-*exo*-methylene group were synthesized in a stereocontrolled manner. The NHK coupling strategy permits the construction of a variety of other *C*-glycosides with modifications within the linker area. Importantly, our biological data have identified **7a** as a new GSL antigen that activates iNKT cells in both mice and humans. Unlike previously reported Th1-polarizing *C*-glycoside analogues, the *exo*-methylene-substituted ligand **7a** not only exhibited its ability to induce cytokines production in human iNKT cells, but also displayed a Th2-biasing profile of cytokines in vivo.^[36] It is striking that two structurally similar antigens, **7a** and **5a**, induce opposite cytokine skewing in vivo. Further studies comparing **5a** and **7a** would be needed to elucidate the mechanism governing their differential effect on the immune response.

Experimental Section

Complete experimental procedure for the synthesis of the GSL analogues, including the mass spectrometry characterization and NMR analysis, are provided in the Supporting Information. Details of the methods for in vitro stimulation of human iNKT cells and FACS plots obtained with mouse splenocytes are also included in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- [1]. For reviews, see: Wennekes T, van den Berg RJBHN, Boot RG, van der Marel GA, Overkleeft HS, Aerts JMFG. *Angew. Chem. Int. Ed.* 2009; 48:8848–8869. Merrill AH Jr. *Chem. Rev.* 2011; 111:6387–6422. [PubMed: 21942574]

- [2]. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. *Science*. 1997; 278:1626–1629. [PubMed: 9374463]
- [3]. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, Fersht AR, Besra GS, Schmidt RR, Jones EY, Cerundolo V. *Nat. Immunol.* 2005; 6:819–826. [PubMed: 16007090]
- [4]. Cerundolo V, Silk JD, Masri SH, Salio M. *Nat. Rev. Immunol.* 2009; 9:28–38. [PubMed: 19079136]
- [5]. Miyamoto K, Miyake S, Yamamura T. *Nature*. 2001; 413:531–534. [PubMed: 11586362]
- [6]. a) Yu KOA, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T, Chang Y-T, Besra GS, Porcelli SA. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:3383–3388. [PubMed: 15722411] b) Forestier C, Takaki T, Im A, Molano JS, Baine I, Jerud ES, Illarionov P, Ndonge R, Howell AR, Santamaria P, Besra GS, DiLorenzo TP, Porcelli SA. *J. Immunol.* 2007; 178:1415–1425. [PubMed: 17237389]
- [7]. Li X, Fujio M, Imamura M, Wu D, Vasan S, Wong C-H, Ho DD, Tsuji M. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:13010–13015. [PubMed: 20616071]
- [8]. a) Trappeniers M, Van Beneden K, Decruy T, Hillaert U, Linclau B, Elewaut D, Van Calenbergh S. *J. Am. Chem. Soc.* 2008; 130:16468–16469. [PubMed: 19554718] b) Tashiro T, Nakagawa R, Inoue S, Shiozaki M, Watarai H, Taniguchi M, Mori K. *Tetrahedron Lett.* 2008; 49:6827–6830.
- [9]. Arora P, Venkataswamy MM, Baena A, Bricard G, Li Q, Veerapen N, Ndonge R, Park JJ, Lee JH, Seo K-C, Howell AR, Chang Y-T, Illarionov PA, Besra GS, Chung S-K, Porcelli SA. *J. Am. Chem. Soc.* 2011; 133:5198–5201. [PubMed: 21425779]
- [10]. Yang G, Schmieg J, Tsuji M, Franck RW. *Angew. Chem. Int. Ed.* 2004; 43:3818–3822.
- [11]. Due to their stability toward enzyme action and a low pH environment, aminocyclohexanes linked to phytoceramide may also be considered as *C*-glycoside-like analogues of **1**: Tashiro T, Nakagawa R, Hirokawa T, Inoue S, Watarai H, Taniguchi M, Moria K. *Tetrahedron Lett.* 2007; 48:3343–3347. Harrak Y, Barra CM, Delgado A, Castaño AR, Llebaria A. *J. Am. Chem. Soc.* 2011; 133:12079–12084. [PubMed: 21728320]
- [12]. Patel O, Cameron G, Pellicci DG, Liu Z, Byun H-S, Beddoe T, McCluskey J, Franck RW, Castaño AR, Harrak Y, Llebaria A, Bittman R, Porcelli SA, Godfrey DI, Rossjohn J. *J. Immunol.* 2011; 187:4705–4713. [PubMed: 21964029]
- [13]. Sullivan BA, Nagarajan NA, Wingender G, Wang J, Scott I, Tsuji M, Franck RW, Porcelli SA, Zajonc DM, Kronenberg M. *J. Immunol.* 2010; 184:141–153. [PubMed: 19949076]
- [14]. Toba T, Murata K, Futamura J, Nakanishi K, Takahashi B, Takemoto N, Tomino M, Nakatsuka T, Imajo S, Goto M, Yamamura T, Miyake S, Annoura H. *Bioorg. Med. Chem.* 2012; 20:2850–2859. [PubMed: 22480852]
- [15]. Li X, Chen G, Garcia-Navarro R, Franck RW, Tsuji M. *Immunology*. 2009; 127:216–225. [PubMed: 19489127]
- [16]. Bricard G, Venkataswamy MM, Yu KO, Im JS, Ndonge RM, Howell AR, Veerapen N, Illarionov PA, Besra GS, Li Q, Chang YT, Porcelli SA. *PLoS One*. 2010; 5:e14374. [PubMed: 21179412]
- [17]. a) Lu X, Song L, Metelitsa LS, Bittman R. *ChemBioChem*. 2006; 7:1750–1756. [PubMed: 17009272] b) Liu Z, Byun H-S, Bittman R. *J. Org. Chem.* 2011; 76:8588–8598. [PubMed: 21958232]
- [18]. a) Takai K, Tagashira M, Kuroda T, Oshima K, Utimoto K, Nozaki H. *J. Am. Chem. Soc.* 1986; 108:6048–6050. [PubMed: 22175376] b) Jin H, Uenishi J, Christ WJ, Kishi Y. *J. Am. Chem. Soc.* 1986; 108:5644–5646.
- [19]. For a review of hydrozirconation and its applications, see: Wipf P, Kendall C. *Topics Organomet. Chem.* 2004; 8:1–25.
- [20]. Wang Z, Shao H, Lacroix E, Wu S-H, Jennings HJ, Zou W. *J. Org. Chem.* 2003; 68:8097. [PubMed: 14535789]
- [21]. Trost BM, Weiss AH, von Wangelin AJ. *J. Am. Chem. Soc.* 2006; 128:8–9. [PubMed: 16390095]
- [22]. Caporusso AM, Lardicci L. *J. Chem. Soc., Perkin Trans. 1*. 1983:949–953.
- [23]. The conditions of decarboxylation were not optimized, and the diyne resulting from Glaser coupling was obtained in about 28% yield.

- [24]. The absolute configuration and *ee* value of **9** were determined by preparation of the (*R*)- and (*S*)-MTPA derivatives; see the Supporting Information.
- [25]. Hart DW, Schwartz J. J. Am. Chem. Soc. 1974; 96:8115–8116.
- [26]. Liu Z, Byun H-S, Bittman R. Org. Lett. 2010; 12:2974–2977. [PubMed: 20518525]
- [27]. Interestingly, changing the molar ratio of **11:15** to 1:2.6 led to the incomplete consumption of **11**, lowering the yield to 63%.
- [28]. Lu X, Arthur G, Bittman R. Org. Lett. 2005; 7:1645–1648. [PubMed: 15816773]
- [29]. For a review of SAE, see: Katsuki T, Martin VS. Org. React. 1996; 48:1–300.
- [30]. Hatakeyama S, Matsumoto H, Fukuyama H, Mukugi Y, Irie H. J. Org. Chem. 1997; 62:2275–2279. [PubMed: 11671543]
- [31]. Liu Z, Bittman R. Org. Lett. 2012; 14:620–623. [PubMed: 22233351]
- [32]. No IL-4 or IFN γ was detected at concentrations lower than 500 ng/mL (data not shown).
- [33]. Teng MWL, Westwood JA, Darcy PK, Sharkey J, Tsuji M, Franck RW, Porcelli SA, Besra GS, Takeda K, Yagita H, Kershaw MH, Smyth MJ. Cancer Res. 2007; 67:7495–7504. [PubMed: 17671220]
- [34]. Fujii S, Shimizu K, Hemmi H, Fukui M, Bonito AJ, Chen G, Franck RW, Tsuji M, Steinman RM. Proc. Natl. Acad. Sci. U.S.A. 2010; 103:11252–11257. [PubMed: 16844772]
- [35]. Liu Y, Goff RD, Zhou D, Mattner J, Sullivan BA, Khurana A, Cantu C, Ravkov EV, Ibegbu CC, Altman JD, Teyton L, Bendelac A, Savage PB. J Immunol. Methods. 2006; 312:34–39. [PubMed: 16647712]
- [36]. To date, only one *C*-glycoside analogue showed Th2 activity in an ex vivo mouse model: Banchet-Cadeddu A, Martinez A, Guillarme S, Parietti V, Monneaux F, Henon E, Renault J-H, Nuzillard J-M, Haudrechy A. Bioorg. Med. Chem. Lett. 2011; 21:2510–2514. [PubMed: 21392982]

Figure 1.

Cytokine production (pg/mL) in human iNKT cells in vitro. Monocytes were pulsed overnight with medium (negative control), 100 ng/mL of **1** or **3** (positive controls), or with 10 μ g/mL of **4** or the ligands **6b**, **7a**, **7b**, **23a**, and **23b**. *** $P < 0.001$, one-way ANOVA.

Figure 2.

In vivo kinetics of the cytokine response. C56Bl/6 mice were injected i.v. with 500 ng of either **1** or **7a**. Time course of IFN γ and IL-4 production after injection of A) **1** and B) **7a**.

Scheme 1.
Structures of KRN7000 (**1**) and analogues.

Scheme 2.
 Synthesis of **12**.

Scheme 3.
Synthesis of **7a** and **7b**.